PROHEXADIONE-CALCIUM (BX-112) Unscheduled DNA Synthesis (§84-2)

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DATA EVALUATION RECORD

STUDY TYPE: Other Genotoxicity: Unscheduled DNA Synthesis in Primary Rat Hepatocytes/Mammalian Cell Cultures
OPPTS Number: 870.5550 OPP Guideline Number: §84-2

DP BARCODE: D246707 SUBMISSION CODE: S543930
P.C. CODE: 112600 TOX. CHEM. NO.: None

TEST MATERIAL (PURITY): Prohexadione-calcium (93.3% a.i.)

SYNONYMS: Calcium salt of 3, 5-dioxo-4-propionyl-cyclohexane-1-carboxylic acid

CITATION: Fautz, R. (1992) Unscheduled DNA Synthesis in Primary Hepatocytes of Male Rats *in vitro* with Prohexadione-Calcium. Cytotest Cell Research GambH & Co. KG, D-6101 Rossdorf, Germany. CCR Project No. 304920. BASF-Project No. 80M0148/929030, BASF Registration Document No. 92/11633. December 3, 1992. MRID 44499904. Unpublished.

SPONSOR: BASF Corporation, Agricultural Products, P.O. Box 13528, Research Triangle Park, NC

EXECUTIVE SUMMARY:

In two independent unscheduled DNA synthesis (UDS) assays (MRID 44499904), primary rat hepatocyte cultures were exposed to prohexadione-calcium (93.3% a.i.) in culture medium at concentrations of 5.00, 16.67, 50.00, 166.67, and 500 µg/mL for 18 hours. The criterion for a positive mutagenic response, as measured by unscheduled DNA synthesis, was a substantial and reproducible, statistically significant increase in the net nuclear grain count, accompanied by a substantial increase in the gross nuclear grain count at any concentration as compared to concurrent negative control values. 2-Acetylaminofluorene (2-AAF), a known inducer of UDS in rat hepatocyte primary cell cultures, served as the positive control.

Prohexadione-calcium was tested up to cytotoxic concentrations, 500 µg/mL. The positive control induced the appropriate response. **There was no evidence that unscheduled DNA synthesis, as determined by radioactive tracer procedures [nuclear silver grain counts] was induced.**

This study is classified as **acceptable (§84-2)** and satisfies the requirements for FIFRA Test Guideline for other genotoxic mutagenicity data.

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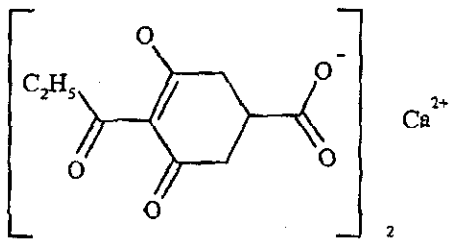
COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: Prohexadione-calcium
   Description: Bright yellow solid
   Lot/Batch #: FW 135
   Purity: 93.3% a.i..
   Stability of compound: Not reported; stored at 4°C in the dark. The study was conducted from July 9 to November 16, 1992.
   CAS #: 127277-53-6
   Structure:



   Vehicle used: Williams medium E (WME)
   Other comments: The test article was dissolved in culture medium on the day of the experiment.

2. Control Materials:
   Negative: Culture medium
   Solvent/final concentration: Not applicable
   Positive (concentrations, solvent): 2-Acetylaminofluorene (2-AAF), dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 2.23 μg/mL in culture medium.

3. Test compound concentrations used: Two preliminary cytotoxicity assays were conducted using 10 concentrations of prohexadione-calcium (0.50, 1.67, 3.33, 5.00, 16.67, 33.33, 50.00, 166.67, 333.33, and 500 μg/mL) dissolved in WME. Based on the results of the preliminary cytotoxicity tests, two independent experiments using the UDS assay were performed at 5 concentrations of prohexadione-calcium (5.00, 16.67, 50.00, 166.67, and 500 μg/mL) dissolved in WME. To determine the toxicity of the test substance, a toxicity test was conducted concurrently with each UDS assay at 8 concentrations of prohexadione-calcium (0.20, 0.50, 1.67, 5.00, 16.67, 50.00, 166.67, and 500 μg/mL) dissolved in WME.

4. Media: Freshly isolated hepatocytes were established in WME supplemented with 2.38 mg/mL of Hepes buffer, 100 U/mL of penicillin, 0.10 mg/mL of streptomycin, 0.29

mg/mL of glutamine, 0.50 µg/mL insulin, and 10% (100 µL/mL) fetal calf serum (FCS). The complete medium was adjusted to pH 7.6.

5. Test Cells: Primary rat hepatocytes were obtained by *in situ*-collagenase perfusion of livers from young adult male Wistar (WU) rats supplied by SAVO, med. Versuchstierzuchten GmbH. The rats were quarantined for at least five days prior to use in the study. They were housed individually in Makrolon Type II cages and fed a pelleted standard diet (Altromin 1324) and tap water ad libitum. The animal room temperature was 21 ± 3° C with 30-93% relative humidity. A 12-hour light/dark cycle was maintained.

6. Cell Preparation:

   a. Perfusion Technique: Rat liver was perfused *in situ* through the hepatic portal vein with Hanks' balanced salt solution supplemented with collagenase (0.5% w/v) adjusted to pH 7.4 and maintained at 37°C.

   b. Hepatocyte Harvest/Culture Preparation: Hepatocytes were isolated from the rat liver and washed twice with perfusion solution without collagenase. The crude cell suspension was filtered through a 94 μm stainless steel mesh to yield a single cell suspension. Viable cells were counted using the trypan blue exclusion method, and the number of isolated cells was determined. The washed hepatocytes were centrifuged and transferred into WME medium. Each well of a six well culture dish containing a Thermanox 25 mm round coverslip was seeded with approximately 1.0×10^5 viable cells/mL in 2.5 mL of WME. Prior to exposure to test compound, vehicle (negative control) or positive control compound, all cultures were incubated for a 1.5 hour attachment period at 37°C in a humidified 5% CO_2 atmosphere. Unattached cells were removed by washing with PBS, and cell numbers and viability (trypan blue exclusion) was determined.

B. TEST PERFORMANCE

1. Preliminary Cytotoxicity Assay: Duplicate cultures were treated with prohexadione-calcium, dissolved in WME medium supplemented with 1% FCS at concentrations of 0.50, 1.67, 3.33, 5.00, 16.67, 33.33, 50.00, 166.67, 333.33, and 500 µg/mL. Duplicate negative controls were included. After 18 hours of exposure at 37°C in the CO_2 (5% CO_2), treated hepatocytes attached to coverslips were rinsed with PBS and incubated with 50 μg/mL of neutral red solution for 3 hours. The solution was discarded and the cultures were rinsed. The dye was eluted with 50% ethanol supplemented with 1% acetic acid. Viability was assessed by the capability of the cells to incorporate the vital dye using a colorimetric absorption method (Fautz, et. al., 1991, Mutation Res. 253:173-179).

2. UDS Assay Concurrent Cytotoxicity Tests: A cytotoxicity test was run concurrently with each UDS assay. After a 1.5 hour attachment period, cells attached to cover slips in 35 mM six-well dishes were fed WME with 1% FCS at prohexadione-calcium dose levels of 0.20, 0.50, 1.67, 5.00, 16.67, 50.00, 166.67, and 500 µg/mL. Two cultures were treated

per dose. Duplicate negative and positive controls were included. The cultures were treated as described for the preliminary cytotoxicity test. Toxicity was determined by the neutral red absorption method.

3. UDS Assay:

a. Treatment: After a 1.5 hour attachment period, cells attached to cover slips in 6-well dishes were fed WME with 1% FCS containing 5 µCi/mL ^{3}H-thymidine (20 Ci/mM) at prohexadione-calcium dose levels of 5.00, 16.67, 50.00, 166.67, and 500 µg/mL. All cultures were in triplicate as were the positive and negative controls. The dishes were incubated for 18 hours at 37°C in the CO_2 incubator. Treated hepatocytes attached to coverslips were then washed twice with PBS, nuclei were swollen by treating hypotonically with 1% sodium citrate (10 minutes), and cells were fixed with three changes of methanol:acetic acid (3:1, v:v) for 20 minutes each. The coverslips were then rinsed with 96% ethanol and air dried. The coverslips were mounted cell side up on slides.

b. Preparation of Autoradiographs/Grain Development: In the dark, the slides were dipped in Ilford K-2 photographic emulsion. The coated slides were stored in light-proof boxes containing drying agent for 7 days at 4°C. Exposed slides were then developed with Kodak D-19 at room temperature and fixed with Tetenal. All slides were then stained with hematoxylin/eosin. The slides were coded prior to grain counting.

c. Grain Counting: Nuclear grains were counted automatically using the Artek 880 or 982 counter for 100 cells (50 cells/2 coverslips) per dose under the oil immersion objective of a Nikon microscope. The mean number of grain counts of one nuclear-sized cytoplasm area adjacent to the nucleus was counted. The net nuclear grain count was determined by subtracting the cytoplasm grain count from the nuclear grain count.

d. Evaluation Criteria: A test article was considered positive if it induced a concentration-related increase of radiolabel incorporation expressed as grains per nucleus or a reproducible and statistically significant positive response for at least one of the dose levels.

e. Statistical Analysis: The non-parametric Mann-Whitney test was reported to be the statistical method of choice for this assay. However, statistical analyses of the data were not performed because the number of nuclear and net grain counts of the groups treated with the test article were in the range of corresponding negative controls.

II. **REPORTED RESULTS**: Concentrations of prohexadione-calcium greater than 166.67 µg/mL formed a precipitate in the culture medium.

A. Preliminary Cytotoxicity Assay: One preliminary cytotoxicity test was conducted to determine the dose range for use in the UDS test and to establish cytotoxic levels of prohexadione-calcium. The test was conducted using prohexadione-calcium at 10 concentrations (0.50-500 μg/mL). The preliminary cytotoxicity results are presented on page 23 of the study report.

The toxicity of prohexadione-calcium in treated cells was determined based on reduced neutral red uptake compared to the negative control. Prohexadione-calcium was cytotoxic at only the highest dose (500 μg/mL); the mean percentage of viable cells relative to the negative control was 51%.

B. UDS Concurrent Cytotoxicity Assays: The toxicity of prohexadione-calcium was determined concurrently with each independent UDS assay at 8 concentrations (0.20-500 μg/mL). The results are summarized on page 24 of the study report and are presented as an Appendix to this DER.

The toxicity of prohexadione-calcium in treated cells was determined based on the percentage viable cells relative to the negative control. Prohexadione-calcium was cytotoxic at 500 μg/mL; the mean percentage of viable cells relative to the negative control was 31%.

In each of the toxicity tests, prohexadione-calcium was cytotoxic only at 500 μg/mL, based on reduced neutral red uptake compared to the negative control. At 500 μg/mL, the mean percentage viable cells was 36% in the first test and 26% in the second test. Percentage viable cells at lower dose levels ranged from 76-120% of the negative control values.

C. UDS Assay: Two independent UDS assays were performed utilizing 5 concentrations of prohexadione-calcium (5-500 µg/mL). Fifty cells per culture were analyzed and two cultures per concentration were evaluated (100 cells/concentration). Appropriate negative (culture medium) and positive (2-AAF) controls were included. The results of the UDS assays were tabulated on pages 26, 27, 29, and 30 of the study report, and the summarized results (study report pages 26 and 29) are presented as an Appendix to this DER. The test material did not induce a higher net nuclear grain count than controls at any treatment level (5-500 μg/mL). In contrast, the positive control induced treatment-related increases in grains/nucleus and net grain counts. Based on these results, prohexadione-calcium failed to show any evidence of DNA-damaging activity in this primary rat hepatocyte UDS assay.

III. REVIEWER'S DISCUSSION/CONCLUSIONS:

A. Based on the results of the preliminary cytotoxicity test, 10 concentrations of prohexadione-calcium (5-500 µg/mL) were selected for the two independent UDS assays. Prohexadione-calcium was cytotoxic at 500 µg/mL, based on the results of the preliminary and concurrent cytotoxicity tests. Prohexadione-calcium is considered negative in the two independent *in vitro* rat primary hepatocyte UDS assays, based on the lack of increased DNA repair synthesis relative to the negative control. The negative control produced the appropriate response and the sensitivity of the system to detect DNA damaging agents was adequately shown by the response induced by the positive control.

B. Study deficiencies - Analyses were not performed to confirm the stability and actual composition of the dosing solutions. However, because the toxicity of prohexadione-calcium to rat hepatocytes in culture was clearly shown, this deficiency is not expected to affect the interpretation of this UDS assay. One other deficiency noted in the study that is not considered to affect the validity of the study results is that historical background rates of 3H-TdR in untreated cell cultures were not provided.

DER - Genotoxicity Review. MRID 44499904

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